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Hyperadherence of an *hha* mutant of *Escherichia coli* O157:H7 is correlated with enhanced expression of LEE-encoded adherence genes ☆

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Abstract

Enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 virulence factors, specifically those conferring intimate adherence to and formation of attaching and effacing lesions (A/E) on host cells, are encoded by a horizontally acquired locus of enterocyte effacement (LEE). Expression of several LEE-encoded genes, which are organized into operons *LEE1* through *LEE5*, is under the positive regulation of *ler*, the first gene in the *LEE1* operon. We have recently demonstrated that EHEC O157:H7 lacking *hha* exhibited greater than a 10-fold increase in *ler* expression and that the repression of *ler* results from the binding of Hha to the *ler* promoter. In this report, we show that an *hha* mutant of EHEC O157:H7 exhibited increased adherence to Hep-2 cells, had increased transcriptional activities of *LEE1*, *LEE2*, *LEE3*, and *LEE5* as determined by reverse transcriptase-polymerase chain reaction assays, and expressed *LEE5::lac* transcriptional fusion at levels that were several-fold higher than that expressed by the parental *hha*$^+$ strain. These results demonstrate that *hha* is an important regulatory component of the cascade that governs the expression of *LEE* operons and the resulting ability of EHEC O157:H7 to intimately adhere to host cells.

Keywords: EHEC; *Escherichia coli* O157:H7; LEE; *hha*

1. Introduction

Enterohemorrhagic *Escherichia coli* (EHEC) O157:H7, a Shiga toxin-producing *E. coli*, is a serious foodborne pathogen causing diarrhea, hemorrhagic colitis (HC), and hemolytic-uremic syndrome (HUS) which can be life threatening [1]. In addition to Shiga toxins that act on vascular endothelial cells to produce HUS, EHEC O157:H7 produces characteristic attaching-and-effacing (A/E) lesions on infected host epithelial cells in experimental animal infection models [2,3]. EHEC and closely related enteropathogenic *E. coli* (EPEC) strains not only produce A/E histopathology in vivo but can also produce A/E lesions when these bacteria adhere to a variety of human epithelial cell lines [4]. Several EHEC strains that were isolated from patients with HC and HUS were shown to produce A/E adherence patterns on Hep-2 cells suggesting that intimate adher-

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ence is an important parameter of EHEC infections [5]. The A/E phenotype requires the concerted action of multiple genes contained within a pathogenicity island, called the locus of enterocyte effacement (LEE) [6].

The genetic organization of LEE from EHEC O157:H7 is similar to that reported for LEE from enteropathogenic *E. coli* (EPEC) O127:H6 [7]. The LEE region of EHEC O157:H7 strain EDL 933 contains 41 ORFs, most of which are organized into five operons named *LEE1* through *LEE5* [8]. The genes within the *LEE1*, *LEE2*, and *LEE3* operons encode for a type III secretion system [9]. The proteins EspA, EspD, and EspB are secreted by the type III secretion system [10,11] and are encoded by the *LEE4* operon. EspA, which forms finger-like projections, facilitates translocation of EspB and Tir to mammalian cells [12]. The genes *eae* and *tir* of *LEE5* encode an outer membrane adhesion protein designated as intimin [13] and a translocated intimin receptor called Tir [14], respectively.

The protein Ler, encoded by the first gene (*ler*) in the *LEE1* operon, is essential for transcriptional activation of *LEE2*, *LEE3*, *LEE4* and *LEE5* operons [15]. Binding of Ler to the upstream (US) regulatory region of *LEE2* is required for the activation of *LEE2* and *LEE3* [16]. Similarly, interactions of Ler with the regulatory region that is located US of *LEE5* operon activates the expression of this operon [17]. Ler has also been shown to counteract the negative regulation exerted by H–NS on the expression of *LEE2* and *LEE3* operons in EPEC [18]. In EPEC, expression of *ler* is regulated by the plasmid encoded Per regulon [19], and quorum-sensing signals are implicated in the density-dependent regulation of *ler* in both EPEC and EHEC [20,21]. The expression of *ler* is increased by the product of the gene *qseA* whose expression is activated by the quorum-sensing signals [22]. We have recently demonstrated that the gene *hha* down-regulates the expression of *ler* in EHEC O157:H7 by binding to the *ler* promoter [23]. Previous studies have also shown that Hha acts as a negative regulator of the hemolysin gene expression in pathogenic *E. coli* [24] and the *inv* gene expression in *Salmonella enterica* serovar Typhimurium [25].

The objective of this study was to compare adherence for an *hha* mutant of EHEC O157:H7 versus a strain containing *hha* and to correlate the magnitude of adherence with the level of expression of *ler*-regulated genes in the mutant strain.

2. Materials and methods

2.1. Bacterial strains, plasmids, and growth conditions

Strains and plasmids used in this study are listed in Table 1. All *E. coli* strains were propagated on Luria–Bertani (LB) agar at 37 °C. For liquid cultures, colonies from LB agar plates were inoculated into LB broth and incubated at 37 °C, unless stated otherwise, in an orbital shaker at 200 rpm. Dulbecco minimal Eagles medium (DMEM) was purchased from Invitrogen, Carlsbad, CA. Media were supplemented, when required, with selective antibiotics at the following concentrations: ampicillin, 50 μg/ml; kanamycin, 50 μg/ml.

2.2. Primer design and PCR amplification

Primers used for PCR amplification of EHEC O157:H7 strain 86–24-specific DNA fragments were selected from the published sequence of EHEC O157:H7 EDL933 [26] and are listed in Table 2. Primers were synthesized by Integrated DNA Technologies (Coralville, IA). PCR amplifications were performed in 50 μl containing 5 μl of DNA (0.2 μg) and 0.3 μM each of forward and reverse primers. AmpliTaq Gold (PE

Table 1
Bacterial strains and plasmids[a]

Strain or plasmid	Relevant genotype and description	Source or reference
E. coli strains		
EHEC 86–24	*stx*$^+$ EHEC strain (serotype O157:H7)	[39]
EHEC Δ*stx2* Δ*lac*	86–24 deleted of *stx2* and *lac* operon	[23]
EHEC *tir::lac*	86–24 Δ*stx2* Δ*lac* containing chromosomal *tir::lac* transcriptional fusion	This study
EHEC *tir::lac* Δ*hha*	86–24 *tir::lac* deleted of *hha*	This study
EHEC 86–24 Δ*hha*	86–24 Δ*stx2* Δ*lac* deleted of *hha*	[23]
TOP 10	*endA1 recA1 hsdR17* ($r_K^- m_K^-$) *sup E44* ϕ*80dlacZ*Δ*M15*Δ(*lacZYA-argF*)	Invitrogen
DH10B	*endA1 recA1 hsdR17* ($r_K^- m_K^-$) *sup E44* ϕ*80dlacZ*Δ*M15*Δ(*lacZYA-argF*)	GIBCO-BRL
Plasmids		
pCR2.1	Cloning vector	Invitrogen
pAM450	Suicide vector	[28]
pSM103	pAM450 derivative used for constructing a *tir::lac* transcriptional fusion	This study
pSM122	pAM450 derivative used for deleting *hha*	[23]
pSM197R	pCR2.1 containing the gene *hha*	This study

[a] Detailed description of bacterial strains and plasmids listed in this table is provided under Section 2.

Table 2
Primers used for PCR

Primer	Nucleotide sequence (5′–3′)	Position/gene/accession number[a]
VS246	CGAGTCGACAGAGATTACAAAGTTGAATCGTCAG	6257–6231/*lacA*/AE005213
VS252	CAGTCTAGAATGTTGTCATCTTTTAATGAC	23181–23161/L0029/AE071034
VS253	CAGGTCGACAAATATCTCCTTTTTATTTTATG	21631–21653/5′ of *tir*/AE071034
VS254	CAGGTCGACATGCCTATTGGTAATCTTGGTC	21630–21609/*tir*/AE071034
VS255	CAGTCTAGAGAGGTGCTAGCCATCGAGCTAC	22308–22329/*tir*/AE071034
VS266	CAGGTCGACAATTTCACACAGGATACAGCTATG	11304–11287*lacZ*/AE005213
VS268	CAAATTGCCACTCACCATTCAG	11100–11121/*lacZ*/AE005213
VS273	TTGGGCTCTAACAGCTCCAGTATCC	21074–21098/*tir*/AE071034
VS274	ACCCGATTGTAGGGATAACCTTGTCA	22065–22039/L0028/AE071034
VS280	CAGGTCGACCCTGATAAGCGAAGCGTATCAGGC	6191–6214/*cynX–lacA* intergenic region/AE005213
VS303	CATCTAGAGATTCCGTTCTCCGTTATGC	3045–3026/*acrB*/AE005213/AE005225
VS305	CAGTCGACACTACTGAACAACATAAAGGTG	1771–1792/*ybaJ*/AE005225
VS306	CAGTCGACTCGCTTTCGGAGCTATAACCG	1409–1388/*ylaD hha* intergenic region/AE005225
VS307	CATCTAGAGCTGGCAGGAGATAAGGAGGT	1–20/downstream of *ylaC*/AE005225
VS309	CAGGATCCCCGGTTATAGCTCCGAAAG	1389–1409/*hha*/AE005225
VS319	CCGTTGAAGTGAAAGACGGTC	1453–1474/*gapA*/AE005401
VS320	AACCACTTTCTTCGCACCAGC	1638–1618/*gapA*/AE005401
VS321	ATACTGTTACCGGCTTTCACG	37583–37563/*escR*/AE071034
VS322	CCAGCCTCCAACAAGAATG	37392–37410/*escR*/AE071034
VS325	TTGTATTTACTGGAGGCCGTG	21176–21156/*tir*/AE071034
VS326	TCTGTGGTGTTTTCCGCAC	20987–21106/*tir*/AE071034
VS340	TGAGCATATTGCGACCTTCG	1882–1864/*ybaJ*/AE005225
VS498	AGTTAGTGGCATCGCCATCAC	30647–30667/*escJ*/AE071034
VS499	GCTGATGACTAAAACGGCTGC	39813–30793/*escJ*/AE071034
VS500	CGCCTGATATCTAAAGCGGTC	27160–27180/*escV*/AE071034
VS501	ACGTTTGGCTTATTGGCTCTG	27337–27316/*escV*/AE071034

[a] Position of the primer sequence represents the location in the published sequence deposited under the indicated accession numbers at NCBI. Underlined sequences GTCGAC and TCTAGA represent restriction sites *Sal*I and *Xba*I, respectively.

Biosystems, Foster City, CA) or Failsafe PCR Kits (Epicenter Technologies, Madison, WI) were used to amplify DNA fragments <2.0 or >2.0-kb, respectively, according to the instructions provided by the manufacturer. PCR amplified products were purified by using either the Qiagen PCR Purification Kit or by agarose gel electrophoresis followed by DNA extraction using a QIAquick Gel Extraction Kit (Qiagen, Valencia, CA).

2.3. Determination of EHEC O157:H7 adherence to Hep-2 cells

Adherence assays were performed as described previously [27]. Hep-2 cells were maintained in RPMI1640 medium supplemented with 10% fetal bovine serum. EHEC O157:H7 86–24 or its derivative strains were grown in LB broth at 37 °C with shaking for 8 h. A 10 μl aliquot of an 8 h bacterial culture was inoculated into 5 ml LB broth and grown statically at 37 °C. Fifty microlitres of static culture containing 1×10^5 bacterial cells was added to the chamber portion of the tissue culture slide (Nalge Nunc International, Naperville, IL) seeded with Hep-2 cells. Slides were incubated at 37 °C for 1 h and then washed four times with phosphate-buffered saline. Slides were immersed in 0.4% crystal violet solution for 15 s, washed with distilled water, air dried, and examined for adherent bacteria at 400× magnification. Assays were performed in triplicate and adherent bacteria were enumerated from 20 Hep-2 cells for each replicate. The significance of the differences in the magnitudes of the adherence displayed by the three strains was assessed using an analysis of variance with Scheffes' *F*-test for multiple comparisons.

2.4. Reverse transcriptase-polymerase chain reaction analysis

Bacterial strains were cultured in LB broth at 37 °C to an OD_{600} of 0.8. Cultures were centrifuged at 6000*g* and cell pellets were processed for RNA isolation using RNAeasy Kit (Qiagen, Valencia, CA) according to the directions of the manufacturer. RNA was treated with 40 units of RNAse-free DNase (Stratagene, La Jolla, CA) at 37 °C and heated to 95 °C for 15 min to inactivate DNase. The RNA was analyzed on an agarose gel to verify its integrity by observing the presence of distinct 23S and 16S rRNA bands, and concentration of RNA determined spectrophotometrically. A single step reverse transcriptase-polymerase chain reaction (RT-PCR) kit (PE Biosystems, Foster City, CA) was used according to the directions of the manufacturer for detecting gene-specific transcripts. The primer sets VS319/VS320, VS321/VS322, VS325/VS326, VS498/VS499, and VS500/VS501 facilitated detection of

transcripts specific to *gapA*, *escR* (*LEE1*), *tir* (*LEE5*), *escJ* (*LEE2*), and *escV* (*LEE3*), respectively. The samples were analyzed on a 4% Nusieve agarose gel containing ethidium bromide (Cambrex Corporation, East Rutherford, NJ) and the gel was visualized and scanned using the spot densitometry software to determine the relative abundance of each amplified DNA band (Alpha Innotech Corporation, San Leandro, CA).

2.5. Construction of tir (LEE5)::lac transcriptional fusion

To introduce *tir::lac* transcriptional fusion in the chromosome of strain 86–24 Δ*stx2* Δ*lac*, a 1.5-kb sequence located upstream (US) and a 1.5-kb sequence located downstream (DS) of the start codon for *tir* was isolated by PCR. The primer sets (VS252-*Xba*I/VS253-*Sal*I and VS254-*Sal*I/VS255-*Xba*I to amplifying US and DS fragments, respectively, for constructing *tir::lac* fusion) used in PCR amplification incorporated restriction sites for *Xba*I at 5′ and *Sal*I at 3′ ends of fragments containing US and *Sal*I at 3′ and *Xba*I at 5′ ends of fragments carrying DS. The 3′ end of the fragment US was joined to the 5′ end of the fragment DS through *Sal*I to generate a 3-kb US–DS fragment. A 5.1-kb *Sal*I fragment containing *lacZ*, *lacY*, and *lacA* ORFs (*lac* cassette) was isolated from strain 86–24 Δ*stx2* *lac*$^+$ by PCR using primers VS266-*Sal*I/VS280-*Sal*I and cloned at the *Sal*I site of US–DS fragment to generate a plasmid pSM99. The 8.1-kb US-*lac*-DS fragment, containing the *lac*Z ORF immediately DS of the *tir* promoter, was isolated from pSM99 using *Xba*I and cloned at the *Xba*I site of pAM450 [28] to produce a plasmid pSM103, which was introduced into strain 86–24 Δ*stx2* Δ*lac* by electroporation. An isolate containing pSM103 was cultured under conditions described previously [23] to facilitate integration and excision events for generating a *tir::lac* transcriptional fusion. The presence of a chromosomal *tir::lac* transcriptional fusion was confirmed by PCR using primers VS268/VS274 and VS273/VS246 to amplify 0.64-kb (*tir* promoter-*lacZ* junction) and 0.623-kb (*tir 5′* sequence-*lacA* junction) fragments, respectively, from the chromosomally generated *tir::lac* fusion. The isolates confirmed for the presence of the *tir::lac* fusion were tested for their β-galactosidase activities.

2.6. Deletion of hha in 86–24 carrying tir::lac transcriptional fusion

The gene *hha* was deleted by using a previously described procedure [23]. Briefly, a 1.3-kb sequence located US and a 1.5-kb sequence located DS of *hha* were isolated by PCR. The primer sets (VS303-*Xba*I/VS305-*Sal*I and VS306-*Sal*I/VS307-*Xba*I for amplifying US and DS fragments, respectively, of *hha*) used in PCR amplification incorporated restriction sites for *Xba*I at 5′ and *Sal*I at the 3′ ends of fragments containing US and *Sal*I at 3′ and *Xba*I at 5′ ends of fragments carrying DS. The 3′ end of the fragment US was joined to the 5′ end of the fragment DS through *Sal*I to generate a 2.8-kb US–DS fragment, which was cloned at the *Xba*I site of pAM450 to generate pSM122. The plasmid pSM122 was introduced into strain 86–24 Δ*stx2* Δ*lac* carrying *tir::lac* fusion, and an isolate containing pSM122 was cultured under conditions to generate *hha* deletion. The presence of *hha* deletion was confirmed by PCR using primers VS309/VS340.

2.7. Determination of β-galactosidase activity

β-galactosidase activity was measured by using the procedure described by Miller [29]. Briefly, an overnight culture was diluted 1:50 in DMEM containing 0.1 M NaCl and 0.45% glucose and grown at 37 °C. Samples were taken at different time intervals to measure optical density at 600 nm (OD_{600}). Aliquots (0.1 ml) were added to a tube containing cell-cracking buffer (25 μl 0.1% sodium dodecyl sulphate, 50 μl chloroform, and 400 μl Z-buffer containing 60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 50 mM β-mercaptoethanol [pH 7.0]). Samples were vortexed and equilibrated to 30 °C for 5 min. After adding an aliquot (0.1 ml) of ONPG (*o*-nitrophenyl-β-D-galactopyranoside), prepared at 4 mg/ml in A-buffer (60 mM K_2HPO_4, 3.3 mM KH_2PO_4, 7.5 mM $(NH_4)_2SO_4$, 0.17 mM sodium citrate), the samples were incubated for additional 20 min at 30 °C. The reactions were stopped by adding 0.25 ml of 1 M Na_2CO_3 and centrifuged (2000*g* for 1 min). Supernatants (100 l) were transferred to a microtiter plate for measuring OD_{420} and OD_{550} using Spectra Max 190 (Molecular Devices, Sunnyvale, CA). β-Galactosidase produced was expressed as units per OD_{600}.

3. Results

3.1. Effect of hha deletion on in vitro adherence of EHEC O157:H7 to Hep-2 cells

We have shown in previous studies that the transcription of *ler* increased greater than 10-fold in EHEC O157:H7 lacking *hha*, which in turn resulted in a 100-fold increase in the expression of *LEE4*-encoded *espA* [23]. Since *espA* along with *espB* and *espD* are critical for the adherence of EHEC O157:H7 to target cells, we determined if enhanced transcription of *ler* and *esp* genes would allow for increased adherence of EHEC O157:H7 to Hep-2 cells. As shown in Fig. 1, the *hha* deletion mutant exhibited significant increase (approximately 3-fold with a p value of <0.0001) in adherence to tissue-cultured cells as compared to the *hha*$^+$ parent

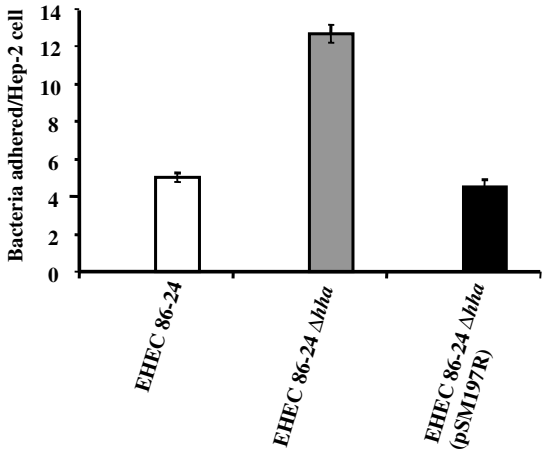

Fig. 1. Adherence of EHEC O157:H7 to Hep-2 cells in the presence or absence of *hha*. Overnight bacterial cultures containing 1×10^5 bacterial cells were added to the chambers of the tissue culture slides that were seeded with Hep-2 cells. Slides were incubated at 37 °C for 1 h, washed with phosphate-buffered saline, and stained with crystal violet solution. Slides were examined for adherent bacteria at 400× magnification. Assays were performed in triplicate in which adherent bacteria were enumerated from 20 Hep-2 cells for each replicate. Error bars indicate standard error of means.

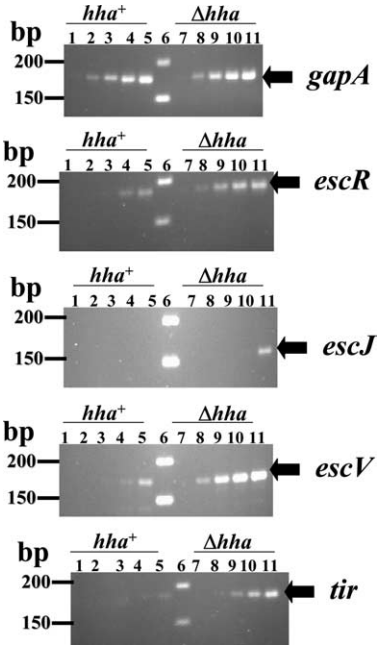

Fig. 2. Determination of transcriptional levels of LEE operons using RT-PCR. Total RNA purified from 86–24 Δ*stx2* Δ*lac* and 86–24 Δ*stx2* Δ*lac* Δ*hha*, which were grown in LB broth at 37 °C (with shaking at 200 rpm), was used in RT-PCR assays containing primer sets for specific amplification and detection of *gapA*-, *escR* (*LEE1*)-, *escV* (*LEE2*)-, *escJ* (*LEE3*)-, and *tir* (*LEE5*)-specific transcripts. The *gapA*, a housekeeping gene, was used as a control. Amplified DNA was resolved on 4% agarose gels containing ethidium bromide and DNA bands were visualized using Alpha Innotech Image documentation system (Alpha Innotech Corporation, San Leandro, CA). Lanes 1 through 5, RT-PCR conducted in the presence of 7.5×10^{-4}, 7.5×10^{-3}, 7.5×10^{-2}, 7.5×10^{-1}, and 7.5 μg of total RNA of 86–24 Δ*stx2* Δ*lac*; lane: 6, DNA size markers (size in bp listed on the left side); lanes 7 through 11, RT-PCR conducted in the presence 7.5×10^{-4}, 7.5×10^{-3}, 7.5×10^{-2}, 7.5×10^{-1}, and 7.5 μg of total RNA of 86–24 Δ*stx2* Δ*lac* Δ*hha*. Arrows on the right point to the position of amplified products specific for *gapA*, *LEE1*, *LEE2*, *LEE3* and *LEE5*.

and the *hha* mutant complemented in *trans* with pSM197R, the plasmid carrying a cloned copy of *hha*. On the other hand, no significant difference was observed in the magnitude of adherence of pSM197R-complemented *hha* mutant (p 0.7705) in comparison to that of the parent *hha*$^+$ strain.

3.2. Expression of ler-regulated LEE operons in hyper-adherent hha mutant

Since intimate adherence of EHEC O157:H7 requires proteins encoded by operons *LEE1* through *LEE5*, one would expect that the expression of these operons might be up-regulated in an *hha* mutant strain that exhibited increased adherence to Hep-2 cells compared to the *hha*$^+$ parent strain. Fig. 2 shows relative amounts of *LEE1*-, *LEE2*-, *LEE3*-, and *LEE5*-specific amplification products that were generated in RT-PCR assays from an EHEC O157:H7 strain carrying or lacking *hha*. Based on the visual examination of the gel, the relative amounts of *gapA*-specific amplification products appeared identical for *hha* and *hha*$^+$ strains at RNA concentrations tested in RT-PCR indicating that the expression of the house keeping gene *gapA* was not affected by the presence or absence of *hha*. On the other hand, samples containing RNA from *hha* strain resulted in amplification of *escR*-, *escJ*-, *escV*-, and *tir*- specific products at 100-fold less RNA template compared to the samples containing RNA from *hha*$^+$ strain. The DNA bands shown in Fig. 2 were also scanned by spot densitometry to determine percent increases in the amounts of amplified products in *hha* mutant strain relative to that produced in *hha*$^+$ strain (Table 3). As shown in this table, *gapA* gene increased by only a 1% in *hha* mutant strain relative to the parent *hha*$^+$ strain at every concentration of RNA tested in RT-PCR. On the other hand, increases of 10% or higher were apparent in amplification products generated for each of the four *LEE* genes in the *hha* mutant strain. These results indicate that enhanced adherence of *hha* mutant strain to Hep-2 cells results from increases in the expression of *LEE* genes.

3.3. Effect of hha deletion on the expression of β-galactosidase activity from a tir::lac fusion

To demonstrate that the enhanced expression of *LEE* operons observed in the *hha* mutant strain was due to increases in the transcriptional activities from the promoters directing the expression of these genes, we constructed a single-copy *tir* (*LEE5*)*::lac* transcriptional fusion in the chromosome of EHEC O157:H7 carrying

Table 3
Expression of LEE genes in *hha* mutant strain

Input RNA (μg) in RT-PCR sample	% Increase in gene expression in *hha*$^-$ strain[a]				
	gapA	*escR*	*escJ*	*escV*	*tir*
7.5	1.12	13.5	44	19.1	41.7
0.75	1.10	14.9	6	19	31.8
0.075	1.17	12.9	ND[b]	19.5	12.9
0.0075	1.22	5.8	ND	7.7	ND
0.00075	ND	ND	ND	ND	ND

[a] Percent increase in the transcription of each gene is given as a ratio of integrated density value of the amplification product produced in *hha* mutant to that produced in *hha*$^+$ parent strain at the equivalent amounts of RNA template added to the RT-PCR mixture.
[b] ND indicates that no detectable levels of amplification products were produced at the indicated amounts of RNA.

or lacking *hha* and monitored the expression of β-galactosidase activities of these fusions. As shown in Fig. 3, the *tir::lac* fusion strain that was lacking *hha* produced significantly higher amounts of β-galactosidase activity (419 units/OD_{600}) after 6 h of growth. On the other hand the *hha*$^+$ EHEC O157:H7 strain carrying *tir::lac* fusion did not produce any detectable levels of β-galactosidase during the 6 h period. Similarly, when pSM197R, pCR2.1 carrying a cloned copy of *hha*, was introduced into an EHEC O157:H7 *tir::lac* strain deleted of the chromosomal copy of *hha*, the expression of β-galactosidase activity was reduced to non-detectable levels that were observed in *hha*$^+$ EHEC O157:H7 *tir::lac* strain.

4. Discussion

A complex cascade of regulatory factors appears to govern the expression of LEE, which encode proteins required by EHEC O157:H7 and EPEC strains to intimately adhere to tissue cultured cells in vitro [30] and to produce the A/E histopathology on intestinal epithelial cells in vivo [6,31]. In EHEC and EPEC, the protein Ler, which is encoded by the first gene of the *LEE1* operon, acts as a positive regulator of *LEE1* through *LEE5* operons [15]. We have recently reported that *hha* represses transcription of *ler* and deletion of *hha* results in the enhanced expression of *ler* and *LEE4* [23]. In this report, we demonstrated that an *hha* mutant strain that hyper-expressed both *ler* and *espA* showed significantly increased adherence to Hep-2 cells and this increase in adherence was correlated with the enhanced expression of *LEE1*, *LEE2*, *LEE3*, and *LEE5* operons. Similarly, the *hha* mutant containing a *tir::lac* fusion produced significantly higher amounts of β-galactosidase activity compared to the *hha*$^+$ or the *hha* mutant strain that was complemented in *trans* with a plasmid-cloned copy of *hha*. Thus, in vitro adherence and transcription data described in this report suggest that *hha* compromises the ability of EHEC O157:H7 for adhering to epithelial cells by reducing the expression of *ler* and *ler* -regulated *LEE* operons.

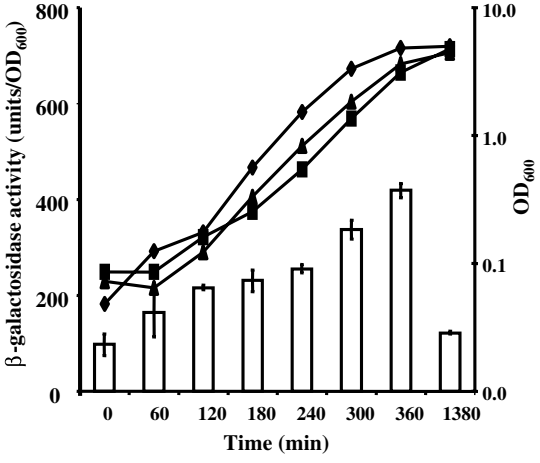

Fig. 3. Expression of β-galactosidase activity. EHEC O157:H7 86–24 *tir::lac*, 86–24 Δ*hha tir::lac*/pCR2.1, and 86–24 Δ*hha tir::lac*/pSM197R were cultured in DMEM containing 0.1 M NaCl at 37 °C (with shaking at 200 rpm) and samples were taken at indicated time intervals for measuring OD_{600} and β-galactosidase activity. OD_{600} is represented as line graphs (86–24 *tir::lac* (◆); 86–24 *hha tir::lac*/pCR2.1 (▲); 86–24 *hha tir::lac*/pSM197R (■). (β-galacatosidase activity of strain 86–24 Δ*hha tir::lac*/pCR2.1 is shown as open bars. Strains 86–24 Δ*hha tir::lac* and 86–24 Δ*hha tir::lac*/pSM197R did not produce any detectable amounts of β-galacatosidase. Error bars indicate standard errors of the means.

Additional regulatory factors that enhance the expression of *ler* have been identified in both EPEC and EHEC. In EPEC, for example, expression of *ler* depends on IHF [32], Fis [33], BipA [34], and PerA, an AraC-like family of transcriptional activators [35]. The quorum-sensing *E. coli* regulator A (QseA) has been shown to activate the expression of *ler* in EPEC and EHEC [22]. Thus, to overcome negative effects exerted by *hha* on the expression of *ler* and LEE-encoded genes both the known (described above) and hitherto unknown positive regulators of *ler* must be expressed to promote increased adherence of EHEC O157:H7 to epithelial cells in the presence of Hha. However, reduced in vitro adherence and expression of LEE genes observed for *hha*$^+$ EHEC strain indicate that one or more of these additional factors required for increased expression of *ler* may not be expressed under in vitro growth conditions. EHEC O157:H7 is capable of producing disease symptoms in the colon at a very low infectious dosage [36], suggesting that intestinal environment provides

essential physico-chemical cues for increased expression of factors that directly and/or indirectly enhance the expression of *ler* and *ler*-dependent LEE genes. The environmental cues such as osmolarity, temperature, pH, oxygen, and ions are some of the important signals that bacterial pathogens use to turn-on or turn-off expression of genes critical for colonization and infection in the host. Recent studies have shown that growth of EHEC O157:H7 in media containing high salt concentrations and incubation temperature of 37 °C, conditions that closely resemble the intestinal environment, induces the expression of *LEE4* operon [37]. It has also been proposed that quorum-sensing signals produced by the normal flora *E. coli* of the large intestine may represent one of the chemical cues for activating LEE in the early stages of infection [20]. In addition, a quorum-sensing regulator (QseA) which is induced by quorum-sensing signals, has been shown to induce the expression of *ler* [22]. Although the nature of the mechanism that governs the regulation of EHEC adherence to host epithelial cells in infectious and pathophysiological states is not completely understood, the results obtained from in vitro gene expression studies suggest that activation of LEE-encoded genes by known and unknown factors is critical to intestinal colonization.

Paradoxically, the expression of Hha is also induced under conditions of high osmolarity [38], suggesting that some of the factors that EHEC O157:H7 expresses in the intestinal environment may either inhibit the binding of Hha to the *ler* promoter or reduce the levels of free Hha to facilitate increased expression of *ler* and *ler*-regulated LEE genes. Thus, it is reasonable to speculate that the expression of these positive regulators of *ler* is enhanced under conditions of high osmolarity, temperature, and quorum-sensing signals to not only counter balance the negative effects of Hha on *ler* expression but to also enhance the expression of *ler* which in turn activates *LEE* operons.

In summary, we have demonstrated that an *hha*$^+$ EHEC O157:H7 strain exhibits reduced adherence to tissue-cultured cells due to reduced expression of *ler* and *ler*-regulated genes. Since expression of *ler* is down-regulated by *hha* and up-regulated by several known factors that are described above, identification of regulatory factors that modulate the levels and/or activity of Hha in relation to positive regulators of *ler* would provide important insights into the pathway governing the adherence of EHEC O157:H7 to mammalian cells.

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